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(54) Title: PRODUCTION AND USE OF NORMALIZED DNA LIBRARIES (57) Abstract Disclosed is a process for forming a normalized genomic DNA library from an environmental sample by (a) isolating a genomic DNA population from the environmental sample; (b) analyzing the complexity of the genomic DNA population so isolated; (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic; and (d) normalizing the representation of various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample. Also disclosed is a normalized genomic DNA library formed from an environmental sample by the process.		

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PRODUCTION AND USE OF NORMALIZED DNA LIBRARIES

The present invention relates to the field of production and screening of gene libraries, and more particularly to the generation and screening of normalized genomic DNA
5 libraries from mixed populations of microbes and/or other organisms.

BACKGROUND OF THE INVENTION

There has been increasing demand in the research reagent, diagnostic reagent and chemical process industries for protein-based catalysts possessing novel capabilities. At present, this need is largely addressed using enzymes purified from a variety of cultivated
10 bacteria or fungi. However, because less than 1% of naturally occurring microbes can be grown in pure culture (Amann, 1995), alternative techniques must be developed to exploit the full breadth of microbial diversity for potentially valuable new products.

Virtually all of the commercial enzymes now in use have come from cultured organisms. Most of these organisms are bacteria or fungi. Amann *et al.* (Amann, 1995) have
15 estimated cultivated microorganisms in the environment as follows:

	<u>Habitat</u>	<u>Culturability (%)</u>
	Seawater	0.001-0.1
	Freshwater	0.25
	Mesotrophic lake	0.01-1.0
20	Unpolluted estuarine waters	0.1-3.0
	Activated sludge	1.0-15.0
	Sediments	0.25
	Soil	0.3

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These data were determined from published information regarding the number of cultivated microorganisms derived from the various habitats indicated.

Other studies have also demonstrated that cultivated organisms comprise only a small fraction of the biomass present in the environment. For example, one group of workers recently reported the collection of water and sediment samples from the "Obsidian Pool" in Yellowstone National Park (Barns, 1994) where they found cells hybridizing to archaea-specific probes in 55% of 75 enrichment cultures. Amplification and cloning of 16S rRNA encoding sequences revealed mostly unique sequences with little or no representation of the organisms which had previously been cultured from this pool, suggesting the existence of substantial diversity of archaea with so far unknown morphological, physiological and biochemical features. Another group performed similar studies on the cyanobacterial mat of Octopus Spring in Yellowstone Park and came to the same conclusion; namely, tremendous uncultured diversity exists (Ward, 1990). Giovannoni *et al.* (1990) and Torsvik *et al.* (1990a) have reported similar results using bacterioplankton collected in the Sargasso Sea and in soil samples, respectively. These results indicate that the exclusive use of cultured organisms in screening for useful enzymatic or other bioactivities severely limits the sampling of the potential diversity in existence.

Screening of gene libraries from cultured samples has already proven valuable. It has recently been made clear, however, that the use of only cultured organisms for library generation limits access to the diversity of nature. The uncultivated organisms present in the environment, and/or enzymes or other bioactivities derived thereof, may be useful in industrial processes. The cultivation of each organism represented in any given environmental sample would require significant time and effort. It has been estimated that in a rich sample of soil, more than 10,000 different species can be present. It is apparent that attempting to individually cultivate each of these species would be a cumbersome task. Therefore, novel methods of efficiently accessing the diversity present in the environment are highly desirable.

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SUMMARY OF THE INVENTION

The present invention addresses this need by providing methods to isolate the DNA from a variety of sources, including isolated organisms, consortias of microorganisms, primary enrichments, and environmental samples, to make libraries which have been
5 "normalized" in their representation of the genome populations in the original samples, and to screen these libraries for enzyme and other bioactivities.

The present invention represents a novel, recombinant approach to generate and screen DNA libraries constructed from mixed microbial populations of cultivated or, preferably, uncultivated (or "environmental") samples. In accordance with the present invention,
10 libraries with equivalent representation of genomes from microbes that can differ vastly in abundance in natural populations are generated and screened. This "normalization" approach reduces the redundancy of clones from abundant species and increases the representation of clones from rare species. These normalized libraries allow for greater screening efficiency resulting in the isolation of genes encoding novel biological
15 catalysts.

Screening of mixed populations of organisms has been made a rational approach because of the availability of techniques described herein, whereas previously attempts at screening of mixed population were not feasible and were avoided because of the cumbersome procedures required.

20 Thus, in one aspect the invention provides a process for forming a normalized genomic DNA library from an environmental sample by (a) isolating a genomic DNA population from the environmental sample; (b) analyzing the complexity of the genomic DNA population so isolated; (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having
25 a desired characteristic; and (d) normalizing the representation of various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample.

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In one preferred embodiment of this aspect, the process comprises the step of recovering a fraction of the isolated genomic DNA having a desired characteristic.

In another preferred embodiment of this aspect, the process comprises the step of amplifying the copy number of the DNA population so isolated.

- 5 In another preferred embodiment of this aspect, the step of amplifying the genomic DNA precedes the normalizing step. In an alternate preferred embodiment of this aspect, the step of normalizing the genomic DNA precedes the amplifying step.

- In another preferred embodiment of this aspect, the process comprises both the steps of
10 (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic.

- Another aspect of the invention provides a normalized genomic DNA library formed from from an environmental sample by a process comprising the steps of (a) isolating a genomic DNA population from the environmental sample; (b) analyzing the complexity
15 of the genomic DNA population so isolated; (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic; and (d) normalizing the representation of various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample. The various
20 preferred embodiments described with respect to the above method aspect of the invention are likewise applicable with regard to this aspect of the invention.

- The invention also provides a process for forming a normalized genomic DNA library from an environmental sample by (a) isolating a genomic DNA population from the environmental sample; (b) analyzing the complexity of the genomic DNA population so
25 isolated; (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired

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characteristic; and (d) normalizing the representation of various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample.

Another aspect of the invention provides a normalized genomic DNA library formed
5 from from an environmental sample by a process comprising the steps of (a) isolating a genomic DNA population from the environmental sample; (b) analyzing the complexity of the genomic DNA population so isolated; (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic; and (d) normalizing the representation of
10 various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample. The various preferred embodiments described with respect to the above method aspect of the invention are likewise applicable with regard to this aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a graph showing the percent of total DNA content represented by G + C in the various genomic DNA isolates tested as described in Example 2.

DETAILED DESCRIPTION OF THE INVENTION

5 DNA ISOLATION:

An important step in the generation of a normalized DNA library from an environmental sample is the preparation of nucleic acid from the sample. DNA can be isolated from samples using various techniques well known in the art (*Nucleic Acids in the Environment Methods & Applications*, J.T. Trevors, D.D. van Elsas, Springer
10 Laboratory, 1995). Preferably, DNA obtained will be of large size and free of enzyme inhibitors and other contaminants. DNA can be isolated directly from the environmental sample (direct lysis) or cells may be harvested from the sample prior to DNA recovery (cell separation). Direct lysis procedures have several advantages over protocols based on cell separation. The direct lysis technique provides more DNA with a generally
15 higher representation of the microbial community, however, it is sometimes smaller in size and more likely to contain enzyme inhibitors than DNA recovered using the cell separation technique. Very useful direct lysis techniques have recently been described which provide DNA of high molecular weight and high purity (Barns, 1994; Holben, 1994). If inhibitors are present, there are several protocols which utilize cell isolation
20 which can be employed (Holben, 1994). Additionally, a fractionation technique, such as the bis-benzimide separation (cesium chloride isolation) described below, can be used to enhance the purity of the DNA.

ANALYSIS OF COMPLEXITY:

Analysis of the complexity of the nucleic acid recovered from the environmental samples
25 can be important to monitor during the isolation and normalization processes. 16S rRNA analysis is one technique that can be used to analyze the complexity of the DNA

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recovered from environmental samples (Reysenbach, 1992; DeLong, 1992; Barns, 1994). Primers have been described for the specific amplification of 16S rRNA genes from each of the three described domains.

FRACTIONATION:

5 Fractionation of the DNA samples prior to normalization increases the chances of cloning DNA from minor species from the pool of organisms sampled. In the present invention, DNA is preferably fractionated using a density centrifugation technique. One example of such a technique is a cesium-chloride gradient. Preferably, the technique is performed in the presence of a nucleic acid intercalating agent which will bind regions
10 of the DNA and cause a change in the buoyant density of the nucleic acid. More preferably, the nucleic acid intercalating agent is a dye, such as bis-benzimide which will preferentially bind regions of DNA (AT in the case of bis-benzimide) (Muller, 1975; Manuelidis, 1977). When nucleic acid complexed with an intercalating agent, such as bis-benzimide, is separated in an appropriate cesium-chloride gradient, the nucleic acid
15 is fractionated. If the intercalating agent preferentially binds regions of the DNA, such as GC or AT regions, the nucleic acid is separated based on relative base content in the DNA. Nucleic acid from multiple organisms can be separated in this manner.

Density gradients are currently employed to fractionate nucleic acids. For example, the use of bis-benzimide density gradients for the separation of microbial nucleic acids for
20 use in soil typing and bioremediation has been described. In these experiments, one evaluates the relative abundance of A_{260} peaks within fixed benzimide gradients before and after remediation treatment to see how the bacterial populations have been affected. The technique relies on the premise that on the average, the GC content of a species is relatively consistent. This technique is applied in the present invention to fractionate
25 complex mixtures of genomes. The nucleic acids derived from a sample are subjected to ultracentrifugation and fractionated while measuring the A_{260} as in the published procedures.

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In one aspect of the present invention, equal A_{260} units are removed from each peak, the nucleic acid is amplified using a variety of amplification protocols known in the art, including those described hereafter, and gene libraries are prepared. Alternatively, equal A_{260} units are removed from each peak, and gene libraries are prepared directly from this
5 nucleic acid. Thus, gene libraries are prepared from a combination of equal amounts of DNA from each peak. This strategy enables access to genes from minority organisms within environmental samples and enrichments, whose genomes may not be represented or may even be lost, due to the fact that the organisms are present in such minor quantity, if a library was construed from the total unfractionated DNA sample. Alternatively,
10 DNA can be normalized subsequent to fractionation, using techniques described hereafter. DNA libraries can then be generated from this fractionated/normalized DNA.

The composition of multiple fractions of the fractionated nucleic acid can be determined using PCR related amplification methods of classification well known in the art.

NORMALIZATION:

- 15 Previous normalization protocols have been designed for constructing normalized cDNA libraries (WO 95/08647, WO 95/11986). These protocols were originally developed for the cloning and isolation of rare cDNA's derived from mRNA. The present invention relates to the generation of normalized genomic DNA gene libraries from uncultured or environmental samples.
- 20 Nucleic acid samples isolated directly from environmental samples or from primary enrichment cultures will typically contain genomes from a large number of microorganisms. These complex communities of organisms can be described by the absolute number of species present within a population and by the relative abundance of each organisms within the sample. Total normalization of each organisms within a
25 sample is very difficult to achieve. Separation techniques such as optical tweezers can be used to pick morphologically distinct members with a sample. Cells from each member can then be combined in equal numbers or pure cultures of each member within

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a sample can be prepared and equal numbers of cells from each pure culture combined to achieve normalization. In practice, this is very difficult to perform, especially in a high thru-put manner.

The present invention involves the use of techniques to approach normalization of the
5 genomes present within an environmental sample, generating a DNA library from the normalized nucleic acid, and screening the library for an activity of interest.

In one aspect of the present invention, DNA is isolated from the sample and fractionated. The strands of nucleic acid are then melted and allowed to selectively reanneal under fixed conditions (C_{ot} driven hybridization). Alternatively, DNA is not fractionated prior
10 to this melting process. When a mixture of nucleic acid fragments is melted and allowed to reanneal under stringent conditions, the common sequences find their complementary strands faster than the rare sequences. After an optional single-stranded nucleic acid isolation step, single-stranded nucleic acid, representing an enrichment of rare sequences, is amplified and used to generate gene libraries. This procedure leads to the
15 amplification of rare or low abundance nucleic acid molecules. These molecules are then used to generate a library. While all DNA will be recovered, the identification of the organism originally containing the DNA may be lost. This method offers the ability to recover DNA from "unclonable sources."

Nucleic acid samples derived using the previously described technique are amplified to
20 complete the normalization process. For example, samples can be amplified using PCR amplification protocols such as those described by Ko *et al.* (Ko, 1990b; Ko, 1990a, Takahashi, 1994), or more preferably, long PCR protocols such as those described by Barnes (1994) or Cheng (1994).

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Normalization can be performed directly, or steps can also be taken to reduce the complexity of the nucleic acid pools prior to the normalization process. Such reduction in complexity can be beneficial in recovering nucleic acid from the poorly represented organisms.

- 5 The microorganisms from which the libraries may be prepared include prokaryotic microorganisms, such as Eubacteria and Archaeobacteria, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. The microorganisms may be cultured microorganisms or uncultured microorganisms obtained from environmental samples and such microorganisms may be extremophiles, such as thermophiles,
10 hyperthermophiles, psychrophiles, psychrotrophs, *etc.*

As indicated above, the library may be produced from environmental samples in which case DNA may be recovered without culturing of an organism or the DNA may be recovered from a cultured organism.

- Sources of microorganism DNA as a starting material library from which target DNA
15 is obtained are particularly contemplated to include environmental samples, such as microbial samples obtained from Arctic and Antarctic ice, water or permafrost sources, materials of volcanic origin, materials from soil or plant sources in tropical areas, etc. Thus, for example, genomic DNA may be recovered from either a culturable or non-culturable organism and employed to produce an appropriate
20 recombinant expression library for subsequent determination of enzyme activity.

- Bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single
25 promoter which initiates transcription of the entire cluster. The gene cluster, the promoter, and additional sequences that function in regulation altogether are referred

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to as an "operon" and can include up to 20 or more genes, usually from 2 to 6 genes. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function.

Some gene families consist of identical members. Clustering is a prerequisite for
5 maintaining identity between genes, although clustered genes are not necessarily identical. Gene clusters range from extremes where a duplication is generated to adjacent related genes to cases where hundreds of identical genes lie in a tandem array. Sometimes no significance is discernable in a repetition of a particular gene. A principal example of this is the expressed duplicate insulin genes in some species,
10 whereas a single insulin gene is adequate in other mammalian species.

It is important to further research gene clusters and the extent to which the full length of the cluster is necessary for the expression of the proteins resulting therefrom. Further, gene clusters undergo continual reorganization and, thus, the ability to create heterogeneous libraries of gene clusters from, for example, bacterial or other
15 prokaryote sources is valuable in determining sources of novel proteins, particularly including enzymes such as, for example, the polyketide synthases that are responsible for the synthesis of polyketides having a vast array of useful activities. Other types of proteins that are the product(s) of gene clusters are also contemplated, including, for example, antibiotics, antivirals, antitumor agents and regulatory proteins, such as
20 insulin.

Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as
25 therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of a huge variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at

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least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating genetic manipulation and *in vitro* studies of these genes/proteins.

The ability to select and combine desired components from a library of polyketides
5 and postpolyketide biosynthesis genes for generation of novel polyketides for study is appealing. The method(s) of the present invention make it possible to and facilitate the cloning of novel polyketide synthases, since one can generate gene banks with clones containing large inserts (especially when using the f-factor based vectors), which facilitates cloning of gene clusters.

10 Preferably, the gene cluster DNA is ligated into a vector, particularly wherein a vector further comprises expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for
15 exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples.

LIBRARY SCREENING:

20 After normalized libraries have been generated, unique enzymatic activities can be discovered using a variety of solid- or liquid-phase screening assays in a variety of formats, including a high-throughput robotic format described herein. The normalization of the DNA used to construct the libraries is a key component in the process. Normalization will increase the representation of DNA from important
25 organisms, including those represented in minor amounts in the sample.

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Example 1

DNA Isolation

1. Samples are resuspended directly in the following buffer:
5 500mM Tris-HCl, pH 8.0
 100mM NaCl
 1mM sodium citrate
 100µg/ml polyadenosine
 5mg/ml lysozyme
- 10 2. Incubate at 37°C for 1 hour with occasional agitation.
3. Digest with 2mg/ml Proteinase K enzyme (Boehringer Mannheim)
 at 37°C for 30 min.
4. Add 8 ml of lysis buffer [200 mM Tris-HCl, pH 8.0/100 mM
15 NaCl/4% (wt/vol) SDS/10% (wt/vol) 4-aminosalicylate] and mix
 gently by inversion.
5. Perform three cycles of freezing in a dry ice-ethanol bath and
 thawing in a 65°C water bath to release nucleic acids.
6. Extract the mixture with phenol and then
 phenol/chloroform/isoamyl alcohol.
- 20 7. Add 4 grams of acid-washed polyvinylpolypyrrolidone (PVPP) to
 the aqueous phase and incubate 30 minutes at 37°C to remove
 organic contamination.
8. Pellet PVPP and filter the supernatant through a 0.45 µm
 membrane to remove residual PVPP.
- 25 9. Precipitate nucleic acids with isopropyl alcohol.
10. Resuspend pellet in 500 µl TE (10 mM Tris-HCl, pH 8.0/1.0 mM
 EDTA)

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11. Add 0.1 g of ammonium acetate and centrifuge mixture at 4°C for 30 minutes.
12. Precipitate nucleic acids with isopropanol.

Example 2

5

Bis-Benzimide Separation of DNA

Sample composed of genomic DNA from *Clostridium perfringens* (27% G+C), *Escherichia coli* (49% G+C) and *Micrococcus lysodictium* (72% G+C) was purified on a cesium-chloride gradient. The cesium chloride (Rf = 1.3980) solution was filtered through a 0.2 µm filter and 15 ml were loaded into a 35 ml OptiSeal tube (Beckman). The DNA was added and thoroughly mixed. Ten micrograms of bis-benzimide (Sigma; Hoechst 33258) were added and mixed thoroughly. The tube was then filled with the filtered cesium chloride solution and spun in a VTi50 rotor in a Beckman L8-70 Ultracentrifuge at 33,000 rpm for 72 hours. Following centrifugation, a syringe pump and fractionator (Brandel Model 186) were used to drive the gradient through an ISCO UA-5 UV absorbance detector set to 280 nm. Three peaks representing the DNA from the three organisms were obtained. PCR amplification of DNA encoding rRNA from a 10-fold dilution of the *E. coli* peak was performed with the following primers to amplify eubacterial sequences:

20

Forward primer: (27F)

5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse primer: (1492R)

5'-GGTTACCTTGTTACGACTT-3'

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Example 3

**Sample of DNA obtained from the gill tissue of a clam
harboring an endosymbiont which cannot be
physically separated from its host**

5

1. Purify DNA on cesium chloride gradient according to published protocols (Sambrook, 1989).
2. Prepare second cesium chloride solution; ($R_f = 1.3980$) filter through 0.2 μ m filter and load 15ml into a 35ml OptiSeal tube (Beckman).
3. Add 10 μ g bis-benzimide (Sigma; Hoechst 33258) and mix.
4. Add 50 μ g purified DNA and mix thoroughly.
5. Spin in a VTi50 rotor in a Beckman L8-70 Ultracentrifuge at 33,000 rpm for 72 hours.
6. Use syringe pump and fractionator (Brandel Model 186) to drive gradient through an ISCO UA-5 UV absorbance detector set to 280nm.

10

15

Example 4

Complexity Analysis

20

1. 16S rRNA analysis is used to analyze the complexity of the DNA recovered from environmental samples (Reysenbach, 1992; DeLong, 1992; Barns, 1994) according to the protocol outlined in Example 1.
2. Eubacterial sequences are amplified using the following primers:
Forward:
5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse:

25

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5'-GGTTACCTTGTTACGACTT-3'

Archaeal sequences are amplified using the following primers:

Forward:

5'-

5 GCGGATCCGCGGCCGCTGCACAYCTGGTYGATYCTGCC-3'

Reverse:

5'-GACGGGCGGTGTGTRCA-3' (R=purine,; Y-
pyrimidine)

3. Amplification reactions proceed as published. The reaction buffer
10 used in the amplification of the archaeal sequences includes 5%
acetamide (Barns, 1994).
4. The products of the amplification reactions are rendered blunt
ended by incubation with *Pfu* DNA polymerase.
5. Blunt end ligation into the pCR-Script plasmid in the presence of
15 *SrfI* restriction endonuclease according to the manufacturer's
protocol (Stratagene Cloning Systems).
6. Samples are sequenced using standard sequencing protocols
(**reference**) and the number of different sequences present in the
sample is determined.

20

Example 5

Normalization

Purified DNA is fractionated according to the bis-benzimide protocol of Example (2),
and recovered DNA is sheared or enzymatically digested to 3-6 kb fragments. Lone-
linker primers are ligated and the DNA is sized selected. Size-selected DNA is
25 amplified by PCR, if necessary.

Normalization is then accomplished as follows:

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1. Double-stranded DNA sample is resuspended in hybridization buffer (0.12 M NaH_2PO_4 , pH 6.8/0.82 M NaCl/1 mM EDTA/0.1% SDS).
- 5 2. Sample is overlaid with mineral oil and denatured by boiling for 10 minutes.
3. Sample is incubated at 68 °C for 12-36 hours.
4. Double-stranded DNA is separated from single-stranded DNA according to standard protocols (Sambrook, 1989) on hydroxyapatite at 60 °C.
- 10 5. The single-stranded DNA fraction is desalted and amplified by PCR.
6. The process is repeated for several more rounds (up to 5 or more).

Example 6

Library Construction

- 15 1. Genomic DNA dissolved in TE buffer is vigorously passed through a 25 gauge double-hubbed needle until the sheared fragments are in the desired size range.
2. DNA ends are "polished" or blunted with Mung Bean nuclease.
- 20 3. *EcoRI* restriction sites in the target DNA are protected with *EcoRI* methylase.
4. *EcoRI* linkers [GGAATTCC] are ligated to the blunted/protected DNA using a very high molar ratio of linkers to target DNA.
5. Linkers are cut back with *EcoRI* restriction endonuclease and the DNA is size fractionated using sucrose gradients.
- 25 6. Target DNA is ligated to the λ ZAPII vector, packaged using *in vitro* lambda packing extracts, and grown in the appropriate *E. coli* XLI Blue host cell.

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Example 7

Library Screening

The following is a representative example of a procedure for screening an expression library prepared in accordance with Example 6.

- 5 The general procedures for testing for various chemical characteristics is generally applicable to substrates other than those specifically referred to in this Example.

- Screening for Activity.** Plates of the library prepared as described in Example 6 are used to multiply inoculate a single plate containing 200 μ L of LB Amp/Meth, glycerol in each well. This step is performed using the High Density Replicating
- 10 Tool (HDRT) of the Beckman Biomek with a 1% bleach, water, isopropanol, air-dry sterilization cycle between each inoculation. The single plate is grown for 2h at 37°C and is then used to inoculate two white 96-well Dynatech microtiter daughter plates containing 250 μ L of LB Amp/Meth, glycerol in each well. The original single plate is incubated at 37°C for 18h, then stored at -80°C. The two condensed daughter
- 15 plates are incubated at 37°C also for 18 h. The condensed daughter plates are then heated at 70°C for 45 min. to kill the cells and inactivate the host E.coli enzymes. A stock solution of 5mg/mL morphourea phenylalanyl-7-amino-4-trifluoromethyl coumarin (MuPheAFC, the 'substrate') in DMSO is diluted to 600 μ M with 50 mM pH 7.5 Hepes buffer containing 0.6 mg/mL of the detergent dodecyl maltoside.

20

MuPheAFC

- Fifty μ L of the 600 μ M MuPheAFC solution is added to each of the wells of the white condensed plates with one 100 μ L mix cycle using the Biomek to yield a final concentration of substrate of ~ 100 μ M. The fluorescence values are recorded (excitation = 400 nm, emission = 505 nm) on a plate reading fluorometer
- 25 immediately after addition of the substrate (t=0). The plate is incubated at 70°C for 100 min, then allowed to cool to ambient temperature for 15 additional minutes. The

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fluorescence values are recorded again (t=100). The values at t=0 are subtracted from the values at t=100 to determine if an active clone is present.

The data will indicate whether one of the clones in a particular well is hydrolyzing the substrate. In order to determine the individual clone which carries the activity, the source library plates are thawed and the individual clones are used to singly inoculate a new plate containing LB Amp/Meth, glycerol. As above, the plate is incubated at 37°C to grow the cells, heated at 70°C to inactivate the host enzymes, and 50 µL of 600 µM MuPheAFC is added using the Biomek. Additionally three other substrates are tested. They are methyl umbelliferone heptanoate, the CBZ-arginine rhodamine derivative, and fluorescein-conjugated casein (~3.2 mol fluorescein per mol of casein).

The umbelliferone and rhodamine are added as 600 µM stock solutions in 50 µL of Hepes buffer. The fluorescein conjugated casein is also added in 50 µL at a stock concentration of 20 and 200 mg/mL. After addition of the substrates the t=0 fluorescence values are recorded, the plate is incubated at 70°C, and the t=100 min. values are recorded as above.

These data indicate which plate the active clone is in, where the arginine rhodamine derivative is also turned over by this activity, but the lipase substrate, methyl umbelliferone heptanoate, and protein, fluorescein-conjugated casein, do not function as substrates.

Chiral amino esters may be determined using at least the following substrates:

For each substrate which is turned over the enantioselectivity value, E, is determined according to the equation below:

$$E = \frac{\ln[(1-c(1+ee_p))]}{\ln[(1-c(1-ee_p))]}$$

- 20 -

where ee_p = the enantiomeric excess (ee) of the hydrolyzed product and c = the percent conversion of the reaction. See Wong and Whitesides, *Enzymes in Synthetic Organic Chemistry*, 1994, Elsevier, Tarrytown, New York, pp. 9-12.

The enantiomeric excess is determined by either chiral high performance liquid
5 chromatography (HPLC) or chiral capillary electrophoresis (CE). Assays are
performed as follows: two hundred μL of the appropriate buffer is added to each well
of a 96-well white microtiter plate, followed by 50 μL of partially or completely
purified enzyme solution; 50 μL of substrate is added and the increase in fluorescence
monitored versus time until 50% of the substrate is consumed or the reaction stops,
10 whichever comes first.

- 21 -

Example 8**Construction of a Stable, Large Insert Picoplankton Genomic DNA Library**

Cell collection and preparation of DNA. Agarose plugs containing concentrated picoplankton cells were prepared from samples collected on an oceanographic cruise from Newport, Oregon to Honolulu, Hawaii. Seawater (30 liters) was collected in Niskin bottles, screened through 10 μm Nitex, and concentrated by hollow fiber filtration (Amicon DC10) through 30,000 MW cutoff polyfulfone filters. The concentrated bacterioplankton cells were collected on a 0.22 μm , 47 mm Durapore filter, and resuspended in 1 ml of 2X STE buffer (1M NaCl, 0.1M EDTA, 10 mM Tris, pH 8.0) to a final density of approximately 1×10^{10} cells per ml. The cell suspension was mixed with one volume of 1% molten Seaplaque LMP agarose (FMC) cooled to 40°C, and then immediately drawn into a 1 ml syringe. The syringe was sealed with parafilm and placed on ice for 10 min. The cell-containing agarose plug was extruded into 10 ml of Lysis Buffer (10mM Tris pH 8.0, 50 mM NaCl, 0.1M EDTA, 1% Sarkosyl, 0.2% sodium deoxycholate, 1 mg/ml lysozyme) and incubated at 37°C for one hour. The agarose plug was then transferred to 40 mls of ESP Buffer (1% Sarkosyl, 1 mg/ml proteinase K, in 0.5M EDTA), and incubated at 55°C for 16 hours. The solution was decanted and replaced with fresh ESP Buffer, and incubated at 55°C for an additional hour. The agarose plugs were then placed in 50 mM EDTA and stored at 4°C shipboard for the duration of the oceanographic cruise.

One slice of an agarose plug (72 μl) prepared from a sample collected off the Oregon coast was dialyzed overnight at 4°C against 1 mL of buffer A (100mM NaCl, 10mM Bis Tris Propane-HCl, 100 $\mu\text{g/ml}$ acetylated BSA: pH 7.0 @ 25°C) in a 2 mL microcentrifuge tube. The solution was replaced with 250 μl of fresh buffer A containing 10 mM MgCl_2 and 1 mM DTT and incubated on a rocking platform for 1 hr at room temperature. The solution was then changed to 250 μl of the same buffer containing 4U of Sau3A1 (NEB), equilibrated to 37°C in a water bath, and then

- 22 -

incubated on a rocking platform in a 37°C incubator for 45 min. The plug was transferred to a 1.5 ml microcentrifuge tube and incubated at 68°C for 30 min to inactivate the enzyme and to melt the agarose. The agarose was digested and the DNA dephosphorylated using Gelase and HK-phosphatase (Epicentre), respectively,
5 according to the manufacturer's recommendations. Protein was removed by gentle phenol/chloroform extraction and the DNA was ethanol precipitated, pelleted, and then washed with 70% ethanol. This partially digested DNA was resuspended in sterile H₂O to a concentration of 2.5 ng/μl for ligation to the pFOS1 vector.

PCR amplification results from several of the agarose plugs (data not shown)
10 indicated the presence of significant amounts of archaeal DNA. Quantitative hybridization experiments using rRNA extracted from one sample, collected at 200 m of depth off the Oregon Coast, indicated that planktonic archaea in (this assemblage comprised approximately 4.7% of the total picoplankton biomass (this sample corresponds to "PACI"-200 m in Table 1 of DeLong *et al.*, high abundance of
15 Archaea in Antarctic marine picoplankton, *Nature*, 371:695-698, 1994). Results from archaeal-biased rDNA PCR amplification performed on agarose plug lysates confirmed the presence of relatively large amounts of archaeal DNA in this sample. Agarose plugs prepared from this picoplankton sample were chosen for subsequent fosmid library preparation. Each 1 ml agarose plug from this site contained
20 approximately 7.5×10^5 cells, therefore approximately 5.4×10^5 cells were present in the 72 μl slice used in the preparation of the partially digested DNA.

Vector arms were prepared from pFOS1 as described (Kim *et al.*, Stable propagation of cosmid sized human DNA inserts in an F factor based vector, *Nucl. Acids Res.*, 20:10832-10835, 1992). Briefly, the plasmid was completely digested with *AstII*,
25 dephosphorylated with HK phosphatase, and then digested with *BamHI* to generate two arms, each of which contained a *cos* site in the proper orientation for cloning and packaging ligated DNA between 35-45 kbp. The partially digested picoplankton DNA was ligated overnight to the pFOS1 arms in a 15 μl ligation reaction containing

- 23 -

25 ng each of vector and insert and 1U of T4 DNA ligase (Boehringer-Mannheim). The ligated DNA in four microliters of this reaction was *in vitro* packaged using the Gigapack XL packaging system (Stratagene), the fosmid particles transfected to *E. coli* strain DH10B (BRL), and the cells spread onto LB_{cm15} plates. The resultant

5 fosmid clones were picked into 96-well microliter dishes containing LB_{cm15} supplemented with 7% glycerol. Recombinant fosmids, each containing ca. 40 kb of picoplankton DNA insert, yielded a library of 3.552 fosmid clones, containing approximately 1.4×10^8 base pairs of cloned DNA. All of the clones examined

10 contained inserts ranging from 38 to 42 kbp. This library was stored frozen at -80°C for later analysis.

Numerous modifications and variations of the present invention are possible in light of the above teachings; therefore, within the scope of the claims, the invention may be practiced other than as particularly described.

- 24 -

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15 Microbiology*, 8:71-81

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Recombinant Biocatalysis, Inc.

(ii) TITLE OF INVENTION: PRODUCTION AND USE OF NORMALIZED DNA LIBRARIES

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: 4225 EXECUTIVE SQUARE, STE. 1400
(C) CITY: LA JOLLA
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 92037

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 INCH DISKETTE
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: WORD PERFECT 6.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Unassigned
(B) FILING DATE: 18 June 1997
(C) CLASSIFICATION: Unassigned

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/665,565
(B) FILING DATE: 18 June 1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 09010/019WO1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 619-678-5070
(B) TELEFAX: 619-678-5099

- 28 -

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 52 NUCLEOTIDES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCGAGAATTC ATTAAAGAGG AGAAATTAAC TATGATTGAA GACCCTATGG AC

52

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 31 NUCLEOTIDES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGAAGATCT TTAAGCACTT CTCTCAGGTT C

31

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 52 NUCLEOTIDES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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52

(2) INFORMATION FOR SEQ ID NO:4:

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(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGAAGATCT TCAGCTAAGC TTCTCTAAGA A

31

- 29 -

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 52 NUCLEOTIDES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCGACAATTG ATTAAAGAGG AGAAATTAAC TATGTGGGAA TTAGACCCTA AA

52

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 31 NUCLEOTIDES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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31

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 52 NUCLEOTIDES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCGACAATTG ATTAAAGAGG AGAAATTAAC TATGACATAC TTAATGAACA AT

52

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 31 NUCLEOTIDES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGAAGATCT TTATGAGAAG TCCCTTTCAA G

31

- 30 -

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 52 NUCLEOTIDES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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52

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- (i) SEQUENCE CHARACTERISTICS
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 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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31

TABLE 1

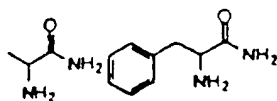
A2

Fluorescein conjugated casein (3.2 mol fluorescein/mol casein)

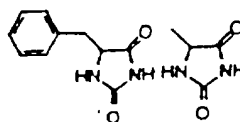
CBZ-Ala-AMC
 t-BOC-Ala-Ala-Asp-AMC
 succinyl-Ala-Gly-Leu-AMC
 CBZ-Arg-AMC
 CBZ-Met-AMC
 morphourea-Phe-AMC

t-BOC = t-butoxy carbonyl, CBZ = carbonyl benzyloxy,
 AMC = 7-amino-4-methyl coumarin

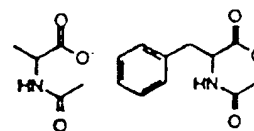
AA3



AB3



AC3



AD3

Fluorescein conjugated casein

t-BOC-Ala-Ala-Asp-AFC
 CBZ-Ala-Ala-Lys-AFC
 succinyl-Ala-Ala-Phe-AFC
 succinyl-Ala-Gly-Leu-AFC

AFC = 7-amino-4-trifluoromethyl coumarin.)

AE3

Fluorescein conjugated casein

AF3

t-BOC-Ala-Ala-Asp-AFC
 CBZ-Asp-AFC

AG3

CBZ-Ala-Ala-Lys-AFC
 CBZ-Arg-AFC

AH3

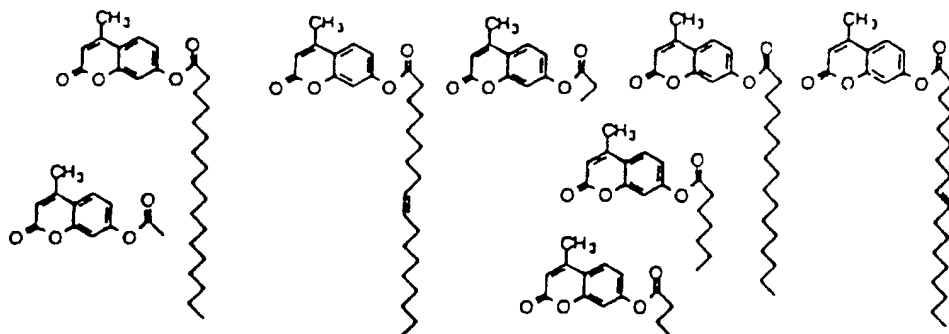
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 CBZ-Phe-AFC
 CBZ-Trp-AFC

AI3

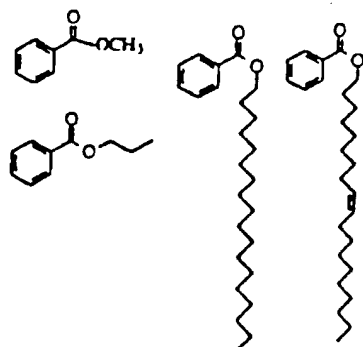
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 CBZ-Ala-AFC
 CBZ-Ser-AFC

TABLE 2

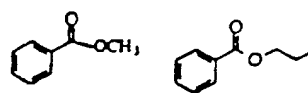
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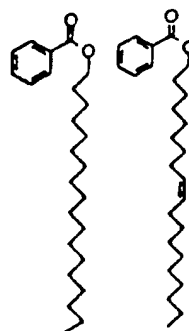
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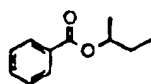
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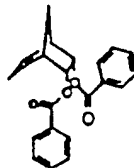
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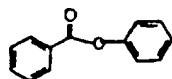
LD3



LF3

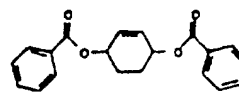


LE3



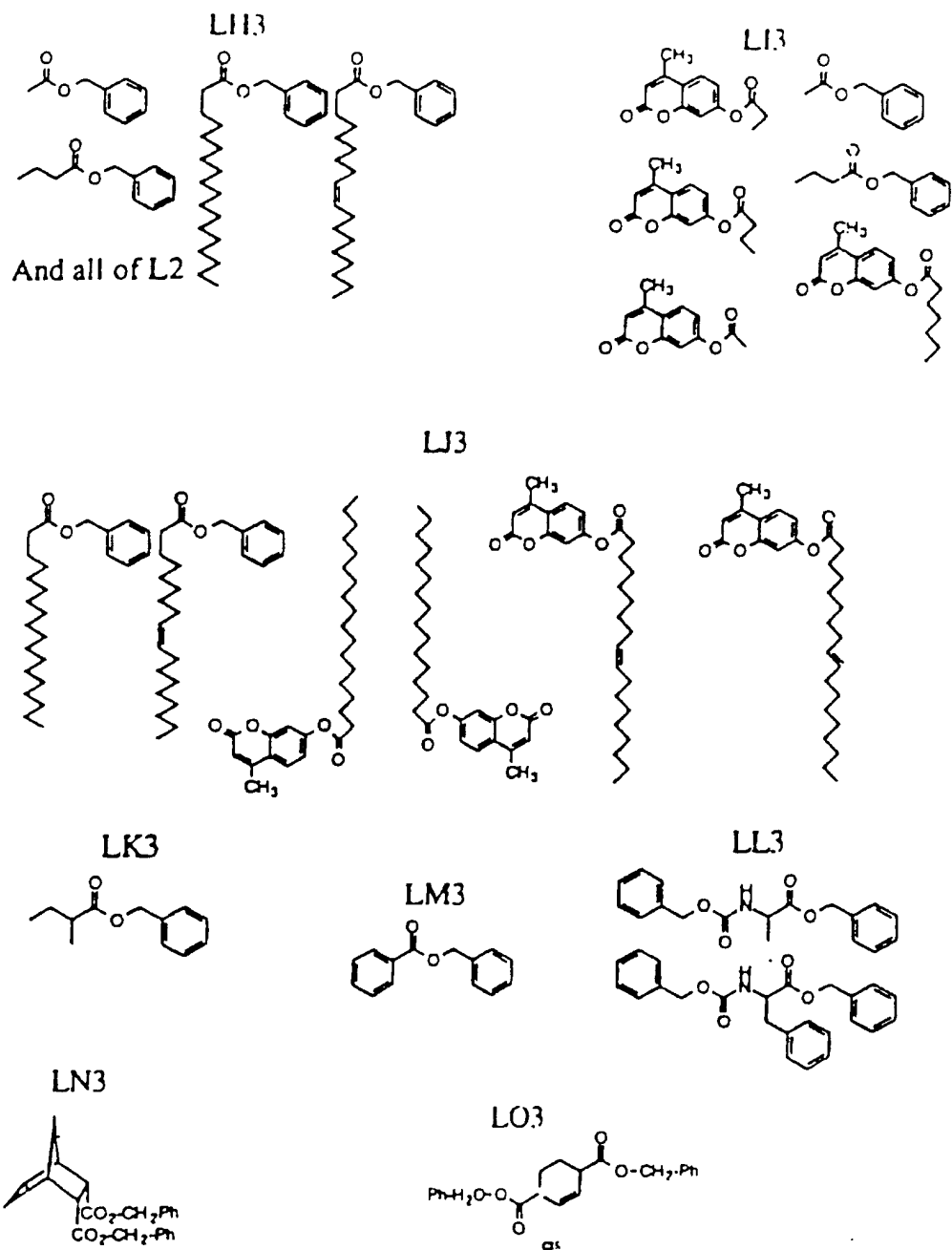
And all of L2

LG3



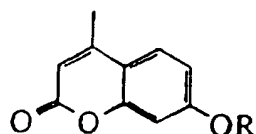
cis

TABLE 3



- 34 -

TABLE 4



4-methyl umbelliferone

wherein R =

G2	β -D-galactose
	β -D-glucose
	β -D-glucuronide
GB3	β -D-cellobioside
	β -D-cellobiopyranoside
GC3	β -D-galactose
	α -D-galactose
GD3	β -D-glucose
	α -D-glucose
GE3	β -D-glucuronide
GI3	β -D-N,N-diacetylchitobiose
GJ3	β -D-fucose
	α -L-fucose
	β -L-fucose
GK3	β -D-mannose
	α -D-mannose

non-Umbelliferoyl substrates

GA3	amylose [polyglucan α 1,4 linkages], amylopectin [polyglucan branching α 1,6 linkages]
GF3	xylan [poly 1,4-D-xylan]
GG3	amylopectin, pullulan
GH3	sucrose, fructofuranoside

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What Is Claimed Is:

1. A process for producing a normalized genomic DNA library from an environmental sample, which comprises the steps of:
 - (a) isolating a genomic DNA population from the environmental sample;
 - 5 (b) analyzing the complexity of the genomic DNA population so isolated;
 - (c) at least one of the steps selected from the group consisting of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic; and
 - (d) normalizing the representation of various DNAs within the genomic DNA
 - 10 population so as to form a normalized library of genomic DNA from the environmental sample.
2. The process of claim 1 which comprises the step of recovering a fraction of the isolated genomic DNA having a desired characteristic.
3. The process of claim 1 which comprises the step of amplifying the copy
- 15 number of the DNA population so isolated.
4. The process of claim 1 wherein the step of amplifying the genomic DNA precedes the normalizing step.
5. The process of claim 1 wherein the step of normalizing the genomic DNA precedes the amplifying step.
- 20 6. The process of claim 1 which comprises both the steps of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic
7. A normalized genomic DNA library formed from from an environmental
- 25 sample by a process comprising the steps of:

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- (a) isolating a genomic DNA population from the environmental sample;
- (b) analyzing the complexity of the genomic DNA population so isolated;
- (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired
5 characteristic; and
- (d) normalizing the representation of various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample.

8. The library of claim 1 wherein the process of forming said library comprises
10 the step of recovering a fraction of the isolated genomic DNA having a desired characteristic.

9. The library of claim 1 wherein the process of forming said library comprises the step of amplifying the copy number of the DNA population so isolated.

10. The library of claim 1 wherein in the process of forming said library the
15 step of amplifying the genomic DNA precedes the normalizing step.

11. The library of claim 1 wherein in the process of forming said library the step of normalizing the genomic DNA precedes the amplifying step.

12. The library of claim 1 wherein the process of forming said library
20 comprises both the steps of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic.

13. A process for forming a normalized library of genomic gene clusters from an environmental sample which comprises
25 (a) isolating a genomic DNA population from the environmental sample;

- 37 -

- (b) analyzing the complexity of the genomic DNA population so isolated;
- (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic; and
- 5 (d) normalizing the representation of various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample.

14. A normalized library of genomic gene clusters formed from from an environmental sample by a process comprising the steps of

- 10 (a) isolating a genomic DNA population from the environmental sample;
- (b) analyzing the complexity of the genomic DNA population so isolated;
- (c) at least one of (i) amplifying the copy number of the DNA population so isolated and (ii) recovering a fraction of the isolated genomic DNA having a desired characteristic; and
- 15 (d) normalizing the representation of various DNAs within the genomic DNA population so as to form a normalized library of genomic DNA from the environmental sample.

1/1

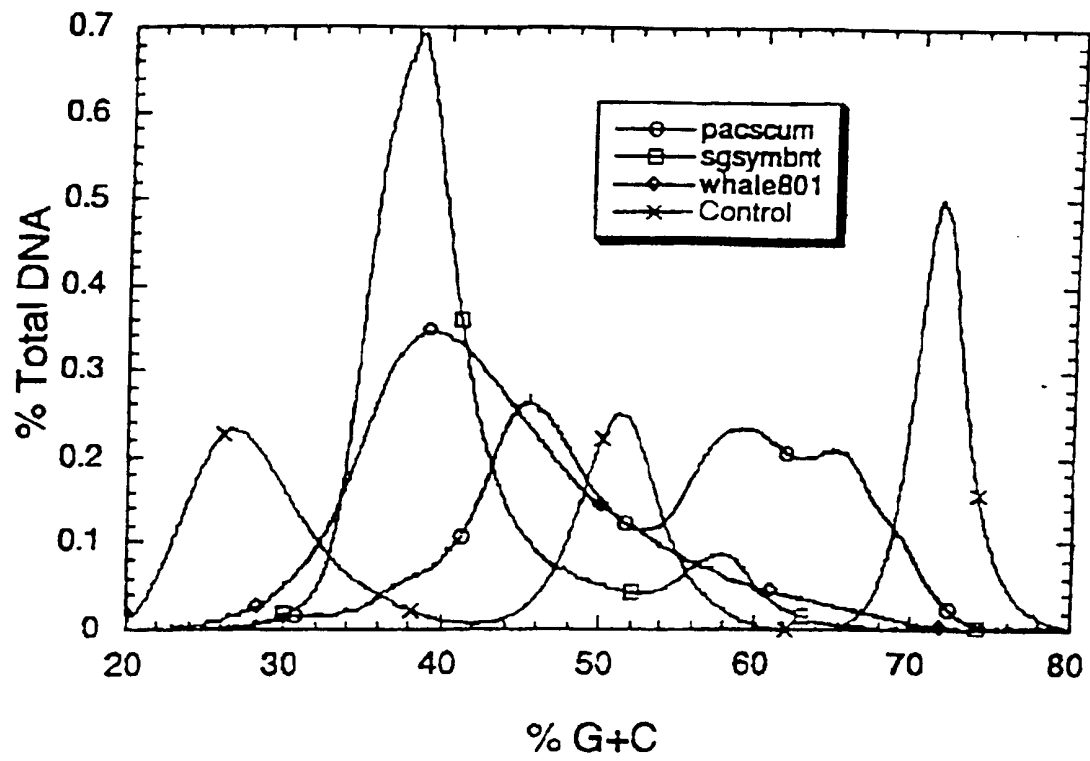


Figure 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10748

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : C07H 21/02, 21/04; C12P 19/34; C12Q 1/68 US CL : 435/6, 91.1; 536/25.4, 25.42 According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/6, 91.1; 536/25.4, 25.42 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, SCISEARCH														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	SIMOENS et al. Isolation of Genes Expressed in Specific Tissues of Arabidopsis thaliana by Differential Screening of a Genomic Library. Gene. 1988, Vol. 6, pages 1-11, see entire document.	7-12												
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"><tr><td>* Special categories of cited documents:</td><td>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td></tr><tr><td>*A* document defining the general state of the art which is not considered to be of particular relevance</td><td>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td></tr><tr><td>*E* earlier document published on or after the international filing date</td><td>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td></tr><tr><td>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td><td>*Z* document member of the same patent family</td></tr><tr><td>*O* document referring to an oral disclosure, use, exhibition or other means</td><td></td></tr><tr><td>*P* document published prior to the international filing date but later than the priority date claimed</td><td></td></tr></table>			* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family	*O* document referring to an oral disclosure, use, exhibition or other means		*P* document published prior to the international filing date but later than the priority date claimed	
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Date of the actual completion of the international search 31 JULY 1997		Date of mailing of the international search report 05 SEP 1997												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer SEAN MCGARRY <i>for</i> Telephone No. (703) 308-0196												